



Metabolic Engineering of Microorganisms for Actinide and Heavy Metal Precipitation

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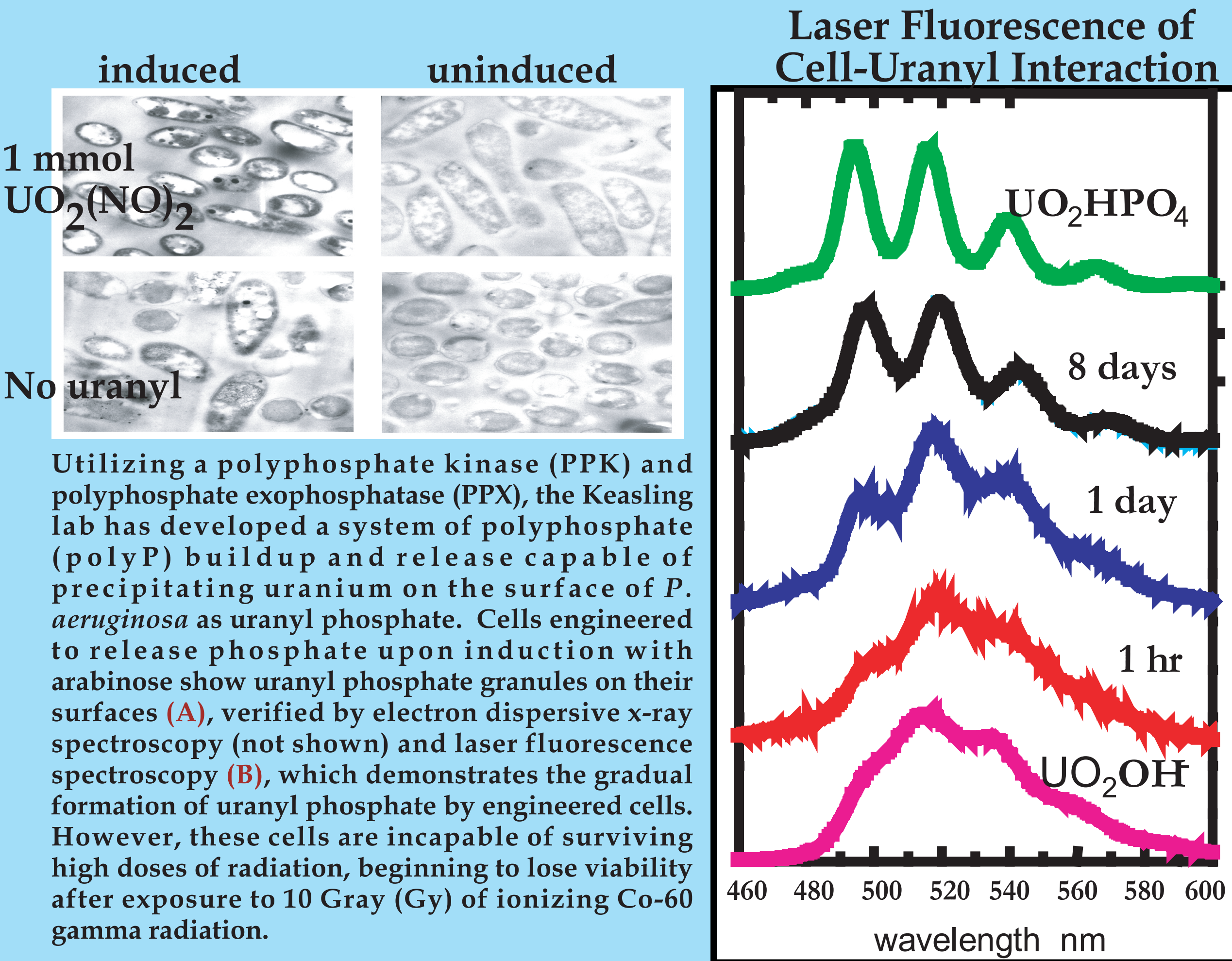
Objectives

The high cost and fragility of current treatment and disposal of radioactive wastes at DOE sites, particularly the transuranic elements, suggest the need for low-cost, mild-condition treatment alternatives. An attractive option is *in situ* bioremediation and biostabilization as well as bioreactor treatment of heavy metals in mixed waste streams. In collaboration with Dr. Mary Lidstrom of the University of Washington, Seattle, we are developing and characterizing strains of the extremely radioresistant bacterium *Deinococcus radiodurans* capable of bioprecipitation of actinides.

The widely reported resistance of *D. radiodurans* to ionizing radiation is exquisitely sensitive to a number of factors, including growth phase, irradiation rate and medium, and growth conditions. There appears to be a correlation between starvation stress and enhanced radiosensitivity; the longer the cells are irradiated for a given dose, the less resistance is observed. Irradiating cells in a nutrient-deficient buffer only affects survivability if cells are subjected to the field for longer than an hour. This variability in radioresistance may have implications for *in situ* bioremediation designs as well as in a bioreactor scenario, as maintaining a simple system is desirable.

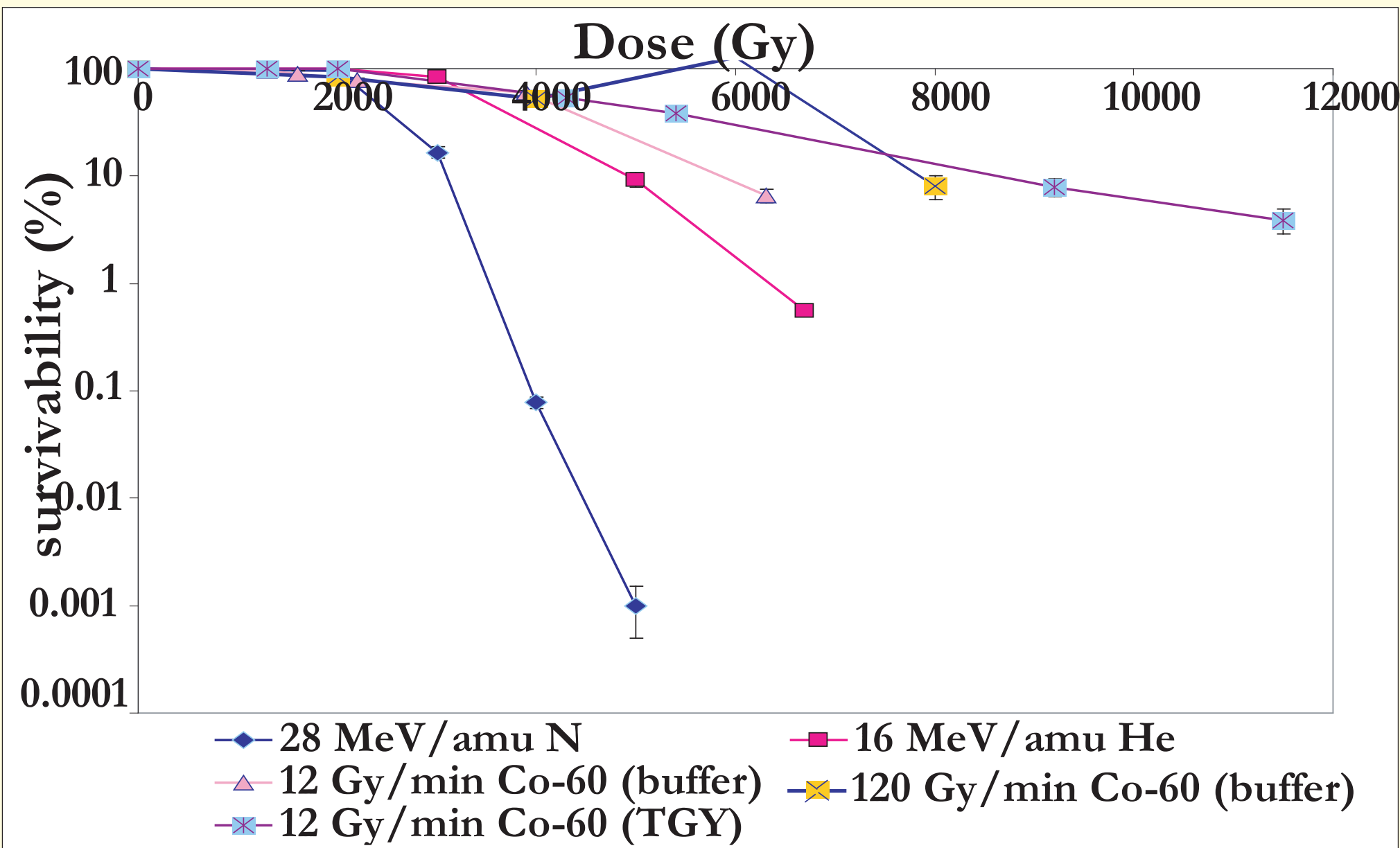
A metabolic construct of *D. radiodurans* developed by a former undergraduate with the Keasling lab, currently at UW Seattle, has been shown to secrete phosphate under mild conditions without induction. However, no phosphate release is seen at the low pHs that typify many waste streams. We also observed an increased rate of phosphate secretion from the engineered strains of both *Pseudomonas aeruginosa* and *D. radiodurans*.

We have characterized the interaction of cells with uranyl chemically and spectroscopically, and data indicate sorption to a surface carboxylate group, most likely from the surface protein layer (S-layer). Working with the Lidstrom group, we will characterize the interaction of uranyl with S-layer knockouts that they are developing and compare them to strain R1. We will also optimize the expression of the phosphate system at lower pHs, which would support a higher uranyl concentration for any potential bioreactor. Finally, we will characterize the engineered *P. aeruginosa* as well as engineered and strain R1 *D. radiodurans* with plutonyl, neptunyl, and curium.



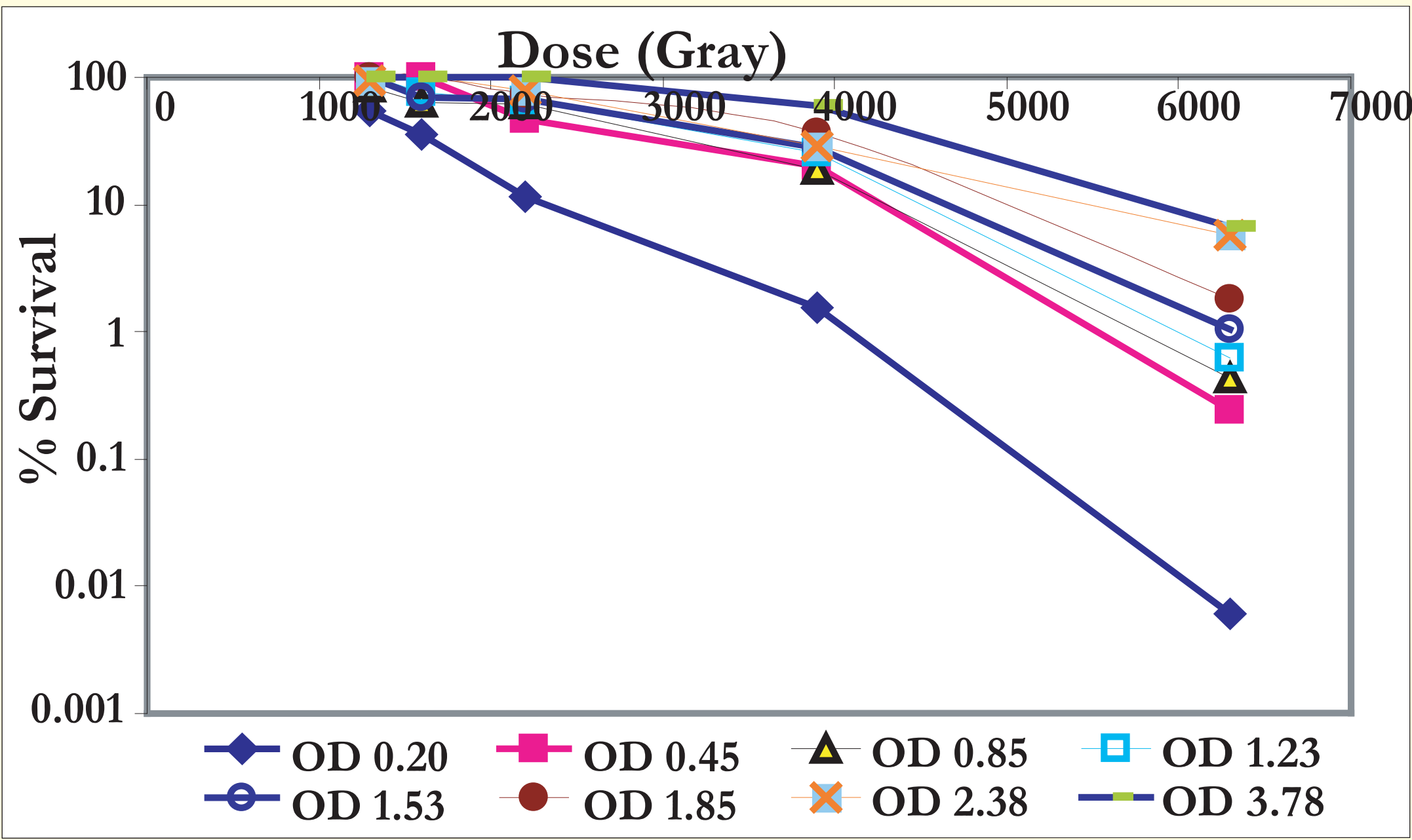
An appealing alternative is *D. radiodurans*, one of the most resistant organisms yet discovered; however, it is sensitive to multiple stresses, has a low native metal resistance, and little or no metal precipitation capacity. Strains of *D. radiodurans* have been engineered to break down organic pollutants, and the Lidstrom group has developed a library of tools for its genetic manipulation. We will combine organic waste remediation capabilities with a similar metal-precipitating phosphate cycle in a single or multiple strain mixed-waste bioremediation system.

D. radiodurans Radioresistance



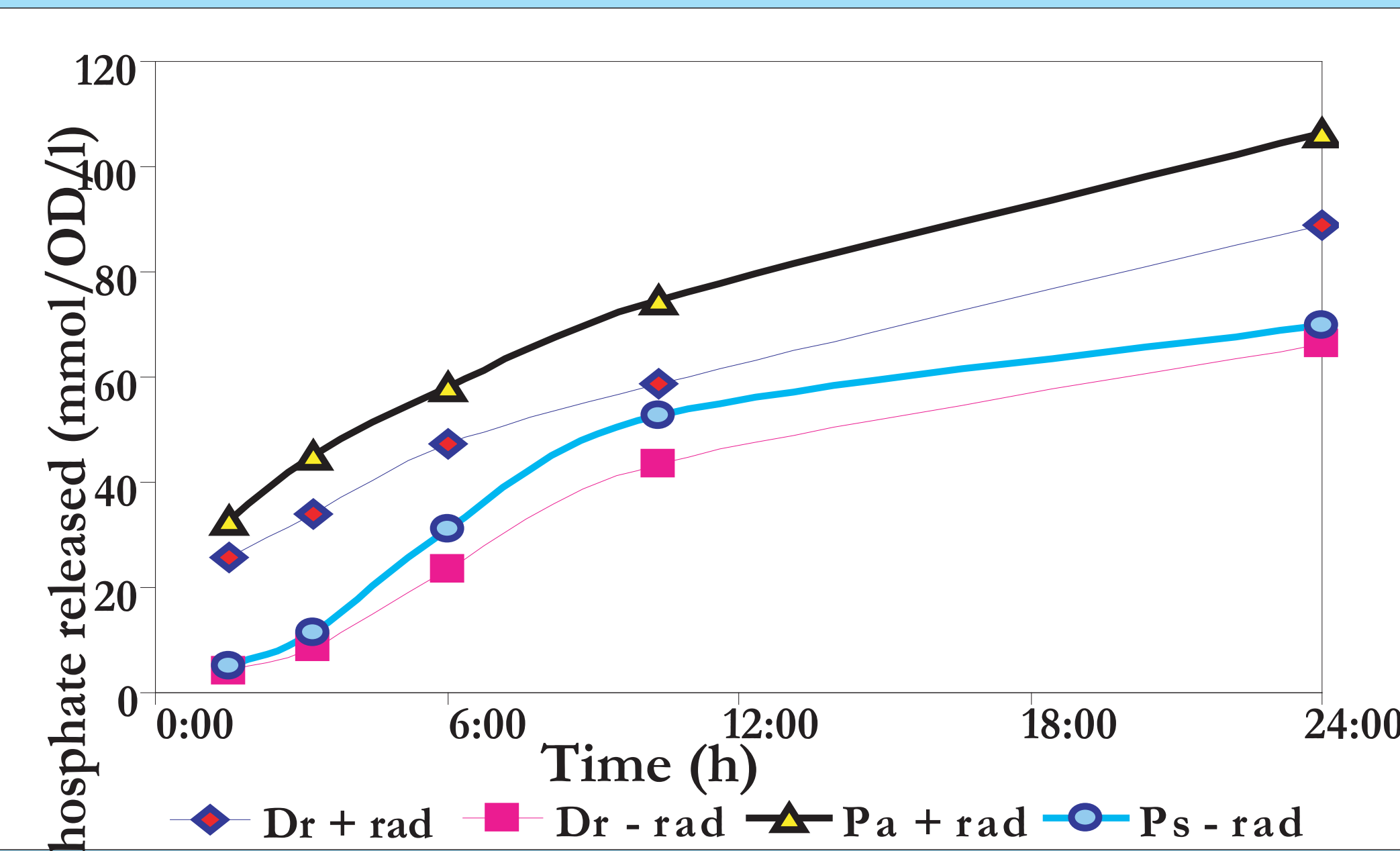
We have observed a marked dependence of radiation resistance on the presence of other stress, most notably starvation in buffer and low oxygen. Ongoing studies with genetic knockouts indicate that certain stress responses are ineffective against simultaneous challenges. As expected, *D. radiodurans* demonstrates increasing radiosensitivity with increasing linear energy transfer (LET) of the ionizing radiation field (C). The bulk of the heavy particle radiation in DOE mixed wastes is due to uranium and plutonium, which emit helium nuclei in the 4-6 MeV range. The studies here were done using LBNL's 88" cyclotron and 16 MeV/amu helium nuclei, the lowest energy that could penetrate the entire length of the irradiation chamber. Although radiation sensitivity increases with LET, the radioresistance of *D. radiodurans* far surpasses any predicted need.

Radiation Survivability and Growth Phase



D. radiodurans demonstrates increasing radioresistance as the culture moves into advanced growth phases, as measured using optical density at 600 nm (OD_{600}) (D). This effect has been noted, but not systematically explored. We attribute this enhanced survivability to a number of factors, including the expression of repair genes in response to age-related stress, the increase in gene copy number, and the recently reported formation of tightly packed toroidal DNA structures, which are thought to enhance radioresistance and increase as the culture ages.

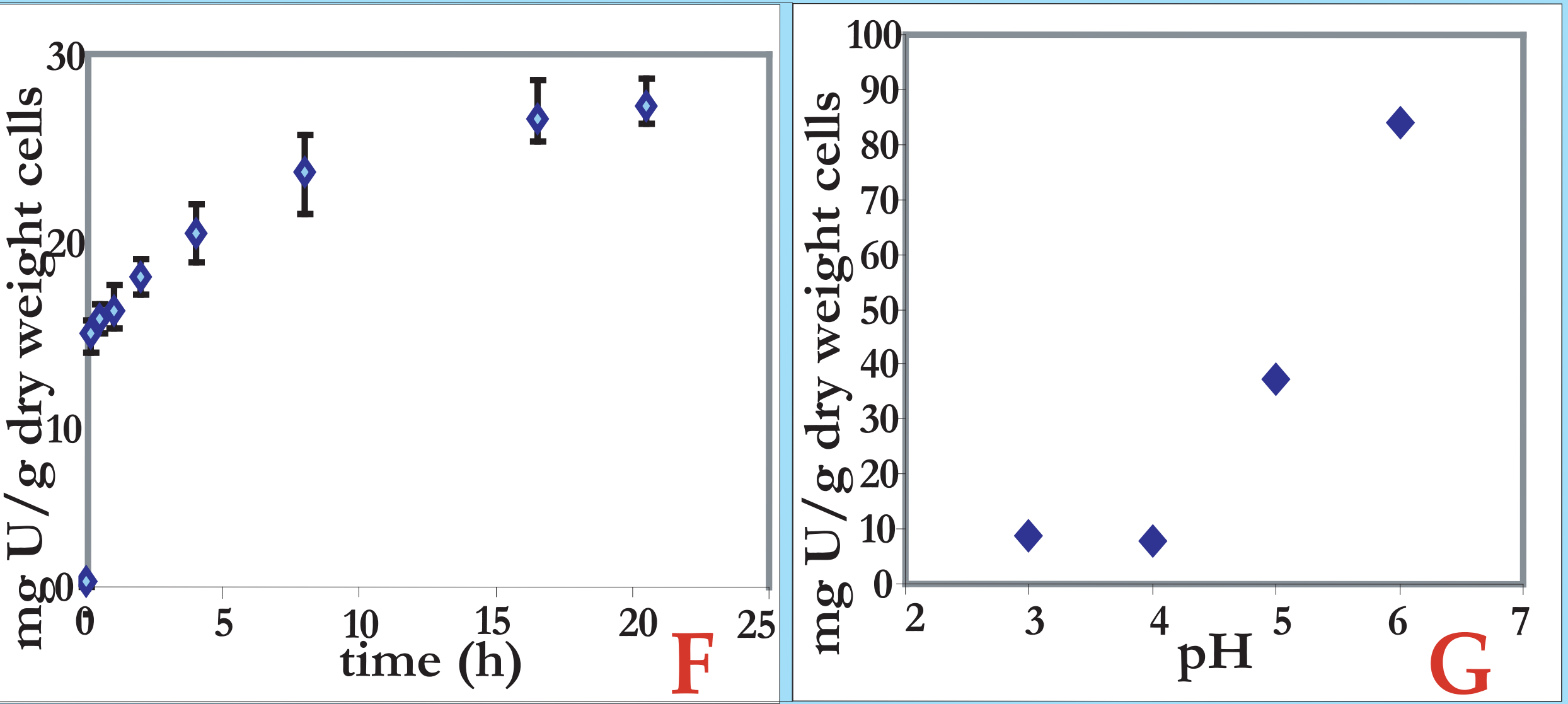
Phosphate Release in Metabolic Constructs



Strains of *D. radiodurans* metabolically engineered in the Lidstrom group release inorganic phosphate upon resuspension in MOPS media (E), indicating that the putative *ppk* gene is capable of being overexpressed on a plasmid and the polyP cycle can be achieved at near-neutral pH. Subjecting to a 40 Gy h^{-1} Co-60 radiation field enhances the rate of phosphate release. We attribute this to the photolytic cleavage of high energy phosphate bonds within the polyP, freeing up ends for the exophosphatase to access. Interestingly, although *P. aeruginosa* is no longer viable after 20 minutes in this radiation field, it too demonstrates an increased rate of phosphate release.

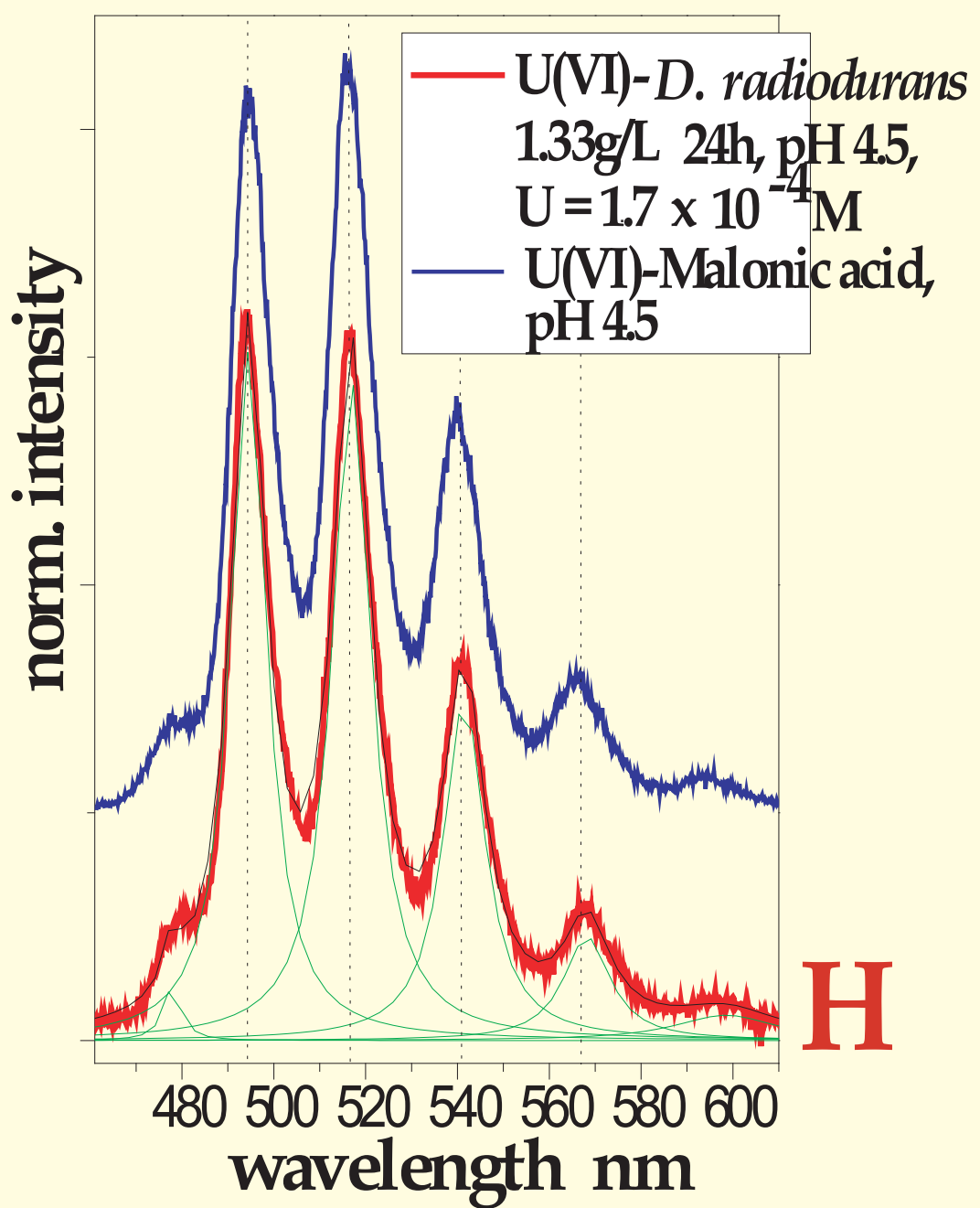
This phosphate release was not observed when the *D. radiodurans* strain was subjected to low pH, however, another example of the varying effects of stress on this microbe. We are currently optimizing low pH phosphate release conditions for this strain as a more stable construct with increased activity is being developed.

Cell-Uranyl Interactions

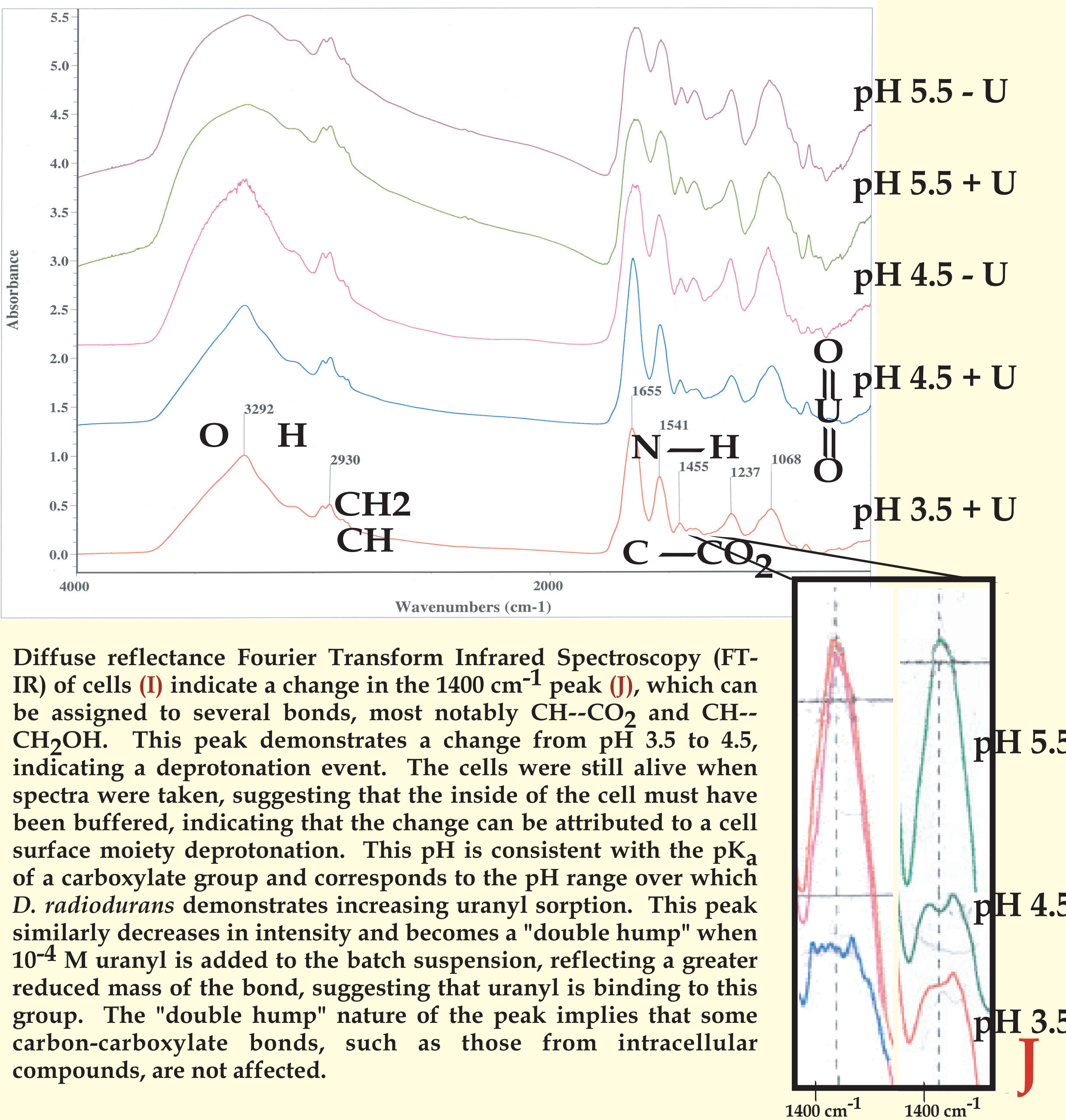


Non-engineered *D. radiodurans* R1 has a very low threshold of metal sorption, approximately fivefold less than *Bacillus sphaericus*, as measured at pH 4.5 and 10^{-4} M uranyl (VI) in 0.1 M NaCl (F). Biomass gradient and live/dead studies (not shown) indicate little or no bioprecipitation of uranyl is occurring. Sorption from a 10^{-6} M uranyl solution at 16 hours increases gradually over several pH units (G), in sharp contrast to the sorption edges seen with other model systems. The pH range over which sorption increases is consistent with typical carboxylic acids, whose pK_a s can range from 4 to 7, indicating uranyl sorption to surface carboxylate groups, most likely from the protein S-layer. We are exploring the binding strength of the uranyl-cell complex using batch experiments with competing ligands to compare to known uranyl-carboxylate moieties. Preliminary results indicate that the surface-sorption complex is extremely weakly bound, underscoring the need for a stable engineered bioprecipitation system.

Spectroscopic Characterization



Spectroscopic studies support the carboxylate hypothesis. Using the Nitsche group's laser spectroscopy facility, we have found that laser fluorescence spectra of the uranyl-cell complex resemble the spectra of a model carboxylate compound (H), in contrast to the uranyl phosphate demonstrated by engineered *P. aeruginosa* (B). In collaboration with other members of the Nitsche group, we plan to definitively confirm the cell-surface carboxylate uranyl interaction using Time Resolved Laser Fluorescence Spectroscopy and building a library of uranyl-carboxylate model compounds. Using our laser facility, we are studying the spectra, decay lifetimes, and binding strengths of model uranyl carboxylates as well as actinide-cell complexes.



We would like to thank Drs. Eleanor Blakely and Kathleen Bjornstad for their help with radiation resistance work and Dr. Hoi-Ying Holman for her aid with the ALS IR beamline. This work was supported by the Natural and Accelerated Bioremediation Research Program (NABIR) in the US Department of Energy, Office of Biological and Environmental Research under Contract No. DE-AC03-76SF00098.